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ORIGINAL INVESTIGATION

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Assignment of the gene responsible for cystinuria (rBAT) and of markers D2S119 and D2S177 to 2p16 by fluorescence in situ hybridization

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Abstract We have established rBAT (named as SLC3A1 in the Genome Data Base) as a gene responsible for cystinuria, a heritable disorder of amino acid transport. The cystinuria locus has been mapped by linkage between microsatellite markers D2S119 and D2S177. Fluorescene in situ hybridization (FISH) either with *Alu*-polymerase-chain-reaction (PCR)-amplified sequences of a yeast artificial chromosome (YAC) containing the rBAT gene or with rBAT-specific PCR-amplified genomic fragments, and chromosome G-banding have cytogenetically mapped rBAT to 2p16.3. In order to correlate the physical and genetic information on cystinuria, we have performed FISH with combinations of *Alu*-PCR- amplified sequences from YACs containing rBAT or the D2S119 and D2S177 loci. In all cases, a fused signal is obtained that demonstrates their close physical location; this allows the assignment of rBAT, cystinuria and their linked markers, D2S119 and D2S177, to 2p16.

Introduction

Cystinuria is a common inherited aminoaciduria disorder that involves the defective transepithelial transport of cystine and dibasic amino acids in the kidney and intestine (MIM 220100, McKusick 1990; Segal and Thier 1989). The cDNA for a human protein (rBAT) involved in the reabsorption of neutral amino acids, cystine and dibasic amino acids in the kidney and intestine has been cloned (Bertran et al. 1993; Lee et al. 1993). Recently, cystinuria-specific mutations in the rBAT gene affecting amino acid transport activity have been reported, demonstrating that this gene is responsible for cystinuria (Calonge et al. 1994). Linkage studies of cystinuria using chromosome 2 markers have localized the cystinuria defect to between D2S119 and D2S177 (Pras et al. 1994).

Somatic cell hybrid studies have localized the human rBAT gene to 2pter-p12 (Calonge et al. 1994; Lee et al. 1993). Further to refine the chromosomal localization of rBAT and of the cystinuria defect, we have identified yeast artificial chromosome (YAC) clones for the loci linked to cystinuria (D2S119 and D2S177) and for the rBAT gene, and we report the localization of these two markers and of the rBAT gene to 2p16.

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Materials and methods

Chromosome preparation

Metaphase spreads were obtained from conventionally cultured human lymphocytes using standard methanol/acetic fixation methods. G-banded chromosomes were photographed, and were destained and refixed according to Klever et al. (1991) before hybridization.

Selection of YAC clones containing the rBAT gene, and D2S119 and D2S177 microsatellite loci

YAC clones from the Centre d'Etude du Polymorphisme Humain (CEPH) MegaYAC library (Albersten et al. 1990) were screened by the polymerase chain reaction (PCR) with primers C1D (Calonge et al. 1994) and PIR 5′-GAACAGCACCTCCTTGGGCAT-3′ (nucleotides 222–202, antisense). To identify the clones containing the whole coding region of the rBAT gene, positive YAC clones were PCR-amplified with three pairs of primers: (1) C1D and PVR 5′-CCATCCTTGTTACTGTCCTT-3′ (nucleotides 412–393, antisense); (2) P3D 5′-GCGTTTGGGGAATCAG-TATG-3′ (nucleotides 1355–1374, sense) and P3R 5′-GTT-CCAGGGAGT-GTGAAAAG-3′ (nucleotides 1425–1406, antisense); (3) P18D 5′-GAGGAATGACAGCCACTATG-3′ (nucleotides 1720–1739,

sense) and P17R 5′-CCAAGCAGCATGCTGTACAT-3′ (nucleotides 2143–2124, antisense) generating fragments corresponding to the 5′, middle and 3′ regions of the rBAT cDNA (Bertran et al. 1993). PCRs to show the presence of the whole coding region of the rBAT gene were performed under standard conditions, with specific YAC DNA encapsulated in agarose beads and obtained as described elsewhere (Overhauser and Radic 1993). YAC clones 668A1 and 761H6 containing markers D2S119 and D2S177, respectively, were obtained by PCR screening the CEPH YAC library (Albersten et al. 1990), using the corresponding primers (Généthon).

Alu-PCR amplifications

Alu-PCR was performed using 3-µl aliquots of bead-DNA, obtained from the corresponding YACs. The reaction mix contained 10 µl buffer (Perkin Elmer Roche), 0.2 m*M* of each of the four dNTPs, 0.45 µ*M* of each primer, *Alu*-5′ (Tagle and Collins 1992) and *Alu* A33 (Chumakov et al. 1992), and 3 U *Taq* polymerase (Perkin Elmer Roche) at a final $MgCl_2$ concentration of 3 m*M*, in a 100-µl final volume. The *Alu*-PCR conditions were those described by Nelson (1990). Aliquots (5 µl) of amplified DNA were analysed on a 1% agarose gel; if suitable, they were ethanol-precipitated and resuspended in 15 µl distilled water before labelling.

Labelling and hybridization with YAC probes

Alu-PCR products from YAC 921B6 containing rBAT were labelled with digoxigenin-11-dUTP (Boehringer Manheim) by a standard nick translation reaction (Selleri et al. 1991), purified by gel filtration and precipitated with NaCl-ethanol and an excess of C_0t-1 DNA (GIBCO-BRL) and salmon sperm DNA (Sigma). The hybridization mix contained 50% formamide and 10% dextran sulphate in 3×SSC (1×SSC = 150 m*M* NaCl/15 m*M* sodium citrate, pH 7.0). Aliquots (10 µl) of the hybridization mix were applied to each slide, covered with a coverslip and sealed with rubber cement. The chromosome preparations and the probe were heat-denatured at 80° C for 8 min. Hybridization took place overnight in a humid chamber at 37° C. Post-hybridization washes were performed as follows: three washes in 50% formamide/2×SSC pH:7 at 43° C, each for 5 min, and three washes in 0.1×SSC at 60° C, each for 5 min. Then, the slides were incubated in blocking solution (Boehringer) for 5 min. Finally, fluorescein-isothiocyanate (FITC)-conjugated sheep anti-digoxigenin (Boehringer Mannheim) was applied, under a coverslip, and the slides were incubated at 37° C for 20 min (Lichter et al. 1988).

Before the slides were mounted, they were washed 3 times in (100 m*M* TRIS-HCl/150 m*M* NaCl)/ 0.05% Tween 20 solution. No signal amplification was performed. Finally, slides were mounted in an antifade solution (Vector Laboratories) containing 0.5 µg/ml propidium iodide.

Alu-PCR products from YACs 668A1 (containing the D2S119 locus) and 761H6 (containing the D2S177 locus) were labelled with biotin-16dUTP by nick translation and hybridized as described above. Hybridization was detected by incubation with FITC-avidin (Vector Laboratories). For post-hybridization washes, we used a 4×SSC/0.1% Tween 20 solution. No rounds of signal amplification were performed. Slides were mounted as described above. For double and triple hybridizations, 7 µl of each of the corresponding hybridization mixtures were used. Hybridization and detection steps were as described above. To re-probe the hybridized slides, we washed and prepared them according to the method described by Heslop-Harrison et al. (1992). In all cases, preparations were studied under an Olympus AH-3 fluorescence microscope equipped with the appropriate filter set.

Labelling and hybridization of rBAT genomic probes

Five different rBAT genomic fragments were PCR-amplified from DNA of a non-cystinuric individual, by adding 3 nmols fluorescein 12-dUTP (Boehringer Mannheim) to the reaction buffer, according to standard methods. The primers used were: C1D (Calonge et al. 1994) and C1Ri 5′-AGAGAGGGCAATGATGGCTA-3′ (nucleotides 333–314, antisense) to generate a 388-bp fragment; C8Di 5′-CCTGAGATGAGATCCAAG-3′ (nucleotides 972–991, sense) and C7Ri 5′-AATGTTCGTGCATTCCGACCT-3′ (nucleotides 1080–1061, antisense) to generate a fragment of approximately 1 kb; iDirA 5′-GACAGCGTCACACAATACTCG-3′ (nucleotides 1012–1032, sense) and C8Ri 5′-TGGCATGTTTTCCATCCAGGA-3′ (nucleotides 1308–1288, antisense) to generate a fragment of approximately 3 kb; C5Di 5′-CCGCAAATGTGAATGAAAGCT-3′ (nucleotides 1472–1492, sense) and C4R (Calonge et al. 1994) to generate a fragment of approximately 1.1 kb; and C9Di 5′-AAGACTCAGCCCAGATCGGC-3′ (nucleotides 1624–1643, sense) and C6R (Calonge et al. 1994) to generate a 488-bp fragment. The nucleotide position refers to the human rBAT cDNA sequence (Bertran et al. 1993). All amplified products were mixed, ethanol-precipitated, and used for hybridization.

The labelled DNA was dissolved in 10 µl hybridization mixture containing 1 µg human C_0t-1 DNA in 50% formamide, 10% dextran sulphate and 1×SSC. The mixture was denatured at 95° C for 10 min. To avoid cross-hybridization, the denatured DNA solution was kept for 20 min at 37° C. The chromosome preparations were denatured separately in 70% formamide/2×SSC at 80° C for 2 min followed by dehydration. Hybridization was carried out at 37° C overnight. The post-hybridization washes were performed in 50% formamide/2×SSC at room temperature for 15 min each. The hybridization signal was amplified three times with anti-fluorescein antibodies and ant-mouse Ig-fluorescein F(ab)2 fragments (Boehringer). During these steps, the chromosome preparations were washed in 4×SSC/0.1% Tween 20 at room temperature for 15 min. After amplification, the preparations were counterstained with propidium iodide (0.2 µg/ml) in antifade solution.

Results

Mapping rBAT

The screening of the CEPH MegaYAC library with primers C1D-PIR revealed specific PCR-amplification products (254 bp) in six YAC clones. Three of these clones (888G9, 921B6, 922B12) contained the whole rBAT cDNA sequence, since specific amplification products of 338 bp, approximately 1.8 kb and 568 bp in length were obtained with three pairs of primers from the 5′-end (C1D-PVR), the middle region (P3D-P3R) and the 3′-end (P18D-P17R), respectively, of the human rBAT cDNA (data not shown).

To localize the human rBAT gene subchromosomally, we performed FISH with the *Alu*-PCR-amplified fragments from one of the rBAT-positive YAC clones (921B6). This YAC was shown not to be chimeric, since only a unique signal was obtained by FISH, mapping to the short arm of chromosome 2 (Fig. 1 A). This localization is in agreement with the previous assignment of the rBAT gene to the short arm of chromosome 2 by somatic cell hybrid analysis (Lee et al. 1993; Calonge et al. 1994) G-banding demonstrated the localization of rBAT in the 2p16 G-band (Fig. 1 B). Confirmation of this assignment and sublocalization of the rBAT gene were performed by G-banding and FISH by using a mixture of five PCR-amplified rBAT genomic fragments as the probe. Our results demonstrate that the rBAT gene maps to 2p16.3 (Fig. 1 C). The cytogenetic localization of the rBAT gene in relation

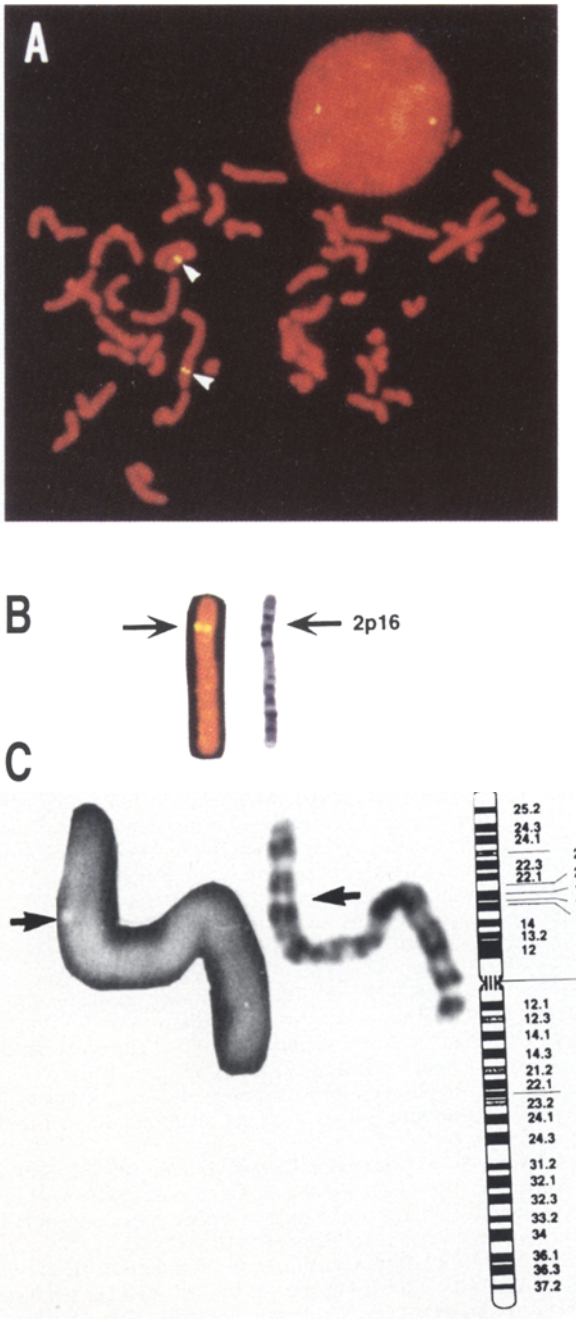

Fig. 1 Localization of the human rBAT gene to chromosome 2p16.3. by fluorescent in situ hybridization either of *Alu*-PCR-amplified YAC clone 921B6, containing the rBAT gene, and detection of the digoxigenin-11-dUTP labelled probe with FITC-conjugated sheep antidigoxigenin (**A, B**) or with rBAT-specific PCR-amplified genomic fragments labelled with fluorescein 12-dUTP (**C**). **A** FISH signals in both 2p regions of a metaphase spread *(arrowheads)* and in an interphase nucleus showing that YAC 921B6 is not chimeric. **B** Chromosome 2 showing the fluorescent hybridization signal *(left)* and G-banding *(right)*. Association of the FISH signal *(arrow)* to the 2p16 band is representative of 30 metaphases from five different hybridizations. **C** Chromosome 2 showing the fluorescent hybridization signal *(left)*, high-resolution G-banding *(middle)* and its G-banded idiogram *(right)*. *Arrows* indicate the hybridization signal and the 2p16.3 band. Association of the FISH signal to the 2p16.3 is representative of 30 metaphases from five different hybridizations. In all cases, chromosomes were counterstained with propidium iodide

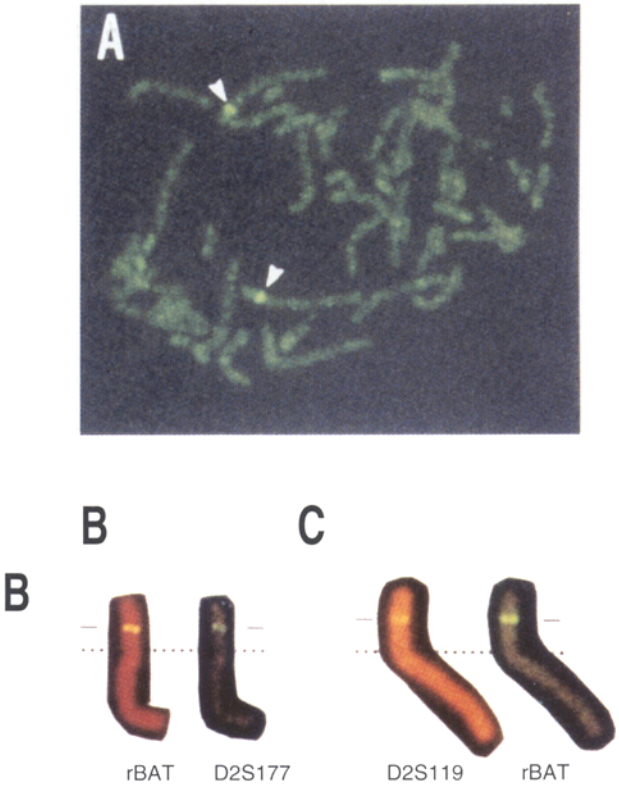

Fig. 2 A–C Propidium iodide counter-staining and fluorescent in situ hybridization of the *Alu*-PCR-amplified YAC clones 921B6, 668A1 and 761H6, containing the rBAT gene, and the D2S119 and D2S177 microsatellite loci, respectively. **A** Metaphase spread showing a single FISH signal *(arrowheads)* on the short arm of chromosome 2 after simultaneous hybridization with the three probes. Digoxigenin-11dUTP-labelled YAC 921B6 probe and biotin-16-dUTP-labelled probes from YACs 668A1 and 761H6 were detected with FITC-conjugated sheep anti-digoxigenin and FITC-avidin, respectively. A single fused signal was obtained in all metaphases analysed from two different hybridizations. **B** Chromosome 2 hybridized first with a probe from YAC 921B6 (rBAT gene) and re-probed with YAC 761H6 (D2S177 marker). Co-localization of both hybridization signals *(bars)* was obtained in all metaphases analysed from a single experiment (the *dotted line* aligns the centromeres). **C** Chromosome 2 first probed with YAC 688A1 (D2S119 marker) and re-probed with YAC 921B6 (rBAT gene). Co-localization of both hybridization signals *(bars)* was obtained in all metaphases analysed from a single experiment (the *dotted line* aligns the centromeres) mapping D2S119 and D2S177

to two markers (D2S119 and D2S177), previously reported to show linkage with the cystinuria locus (Pras et al. 1994), was studied. To this end, combinations of *Alu*-PCR-amplified sequences from YACs 668A1, 761H6 and 921B6, containing microsatellite loci D2S119 and D2S177, and the rBAT gene, respectively, were used for triple and double fluorescence hybridizations. In all cases, triple (Fig. 2 A) and double hybridizations (668A1/761H6, 668A1/921B6 and 761H6/921B6) (data not shown) gave a single fused signal in the p16 G-band of chromosome 2. These results demonstrate the close physical location of the three markers. To demonstrate this co-localization further, chromosome metaphases were first hybridized with *Alu*-PCR-amplified sequences of YACs 921B6 (rBAT

gene) or 668A1 (microsatellite loci D2S119), and after de-hybridization, the same samples were re-probed, substituting 921B6 for 761H6 (microsatellite loci D2S177) and 668A1 for 921B6 (rBAT gene). In all cases, the FISH signals were localized to the same chromosome 2 band (Fig. 2 B and C).

Discussion

The present results demonstrate that the human rBAT gene maps to band 2p16.3 and that it lies in close vicinity to the D2S119 and D2S177 loci. This localization is based on FISH analysis of high resolution trypsin-Giemsa-banded chromosomes. During the course of our study, Udenfriend's group has reported that the human rBAT (also named NBAT) gene is localized to the contiguous band 2p21, as revealed by 4′, 6-diamidino-2-phenylindole staining (Yan et al. 1994). Other than the different level of G-banding resolution and the staining method, we have no explanation for this discrepancy.

The rBAT gene has been demonstrated to be responsible for the cystinuria phenotype: cystinuria-specific mutations in the rBAT gene, found in Spanish and Italian families, affect the amino acid transport activity of rBAT when the protein is expressed in *Xenopus* oocytes (Calonge et al. 1994). Linkage studies between cystinuria and chromosome 2p markers in Middle Eastern families have established the cystinuria locus to be approximately 7 centiMorgans (cM) telomeric to D2S119, between D2S119 and D2S177 (Pras et al. 1994). In a recent high resolution genetic map, these loci have been assigned to band 2p15 (D2S119) and to the centromeric end of band 2p16 (D2S177), with a genetic distance of 5 cM between them (Matise et al. 1994). Our FISH studies demonstrate that the rBAT gene and its two flanking markers co-localize physically to band 2p16. This result is in full agreement with linkage studies and further confirms that the rBAT gene corresponds to the assigned cystinuria locus. The cytogenetic and genetic localization of the gene responsible for cystinuria (rBAT) introduces a new reference on the short arm of chromosome 2 for further genetic mapping.

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